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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/52, 9/00, C07H 21/04, A61K 31/70 A1

(11) International Publication Number: WO 95/11304

(43) International Publication Date: 27 April 1995 (27.04.95)

(21) International Application Number: PCT/US94/11649

(22) International Filing Date: 14 October 1994 (14.10.94)

(30) Priority Data:
08/139,176 18 October 1993 (18.10.93) US

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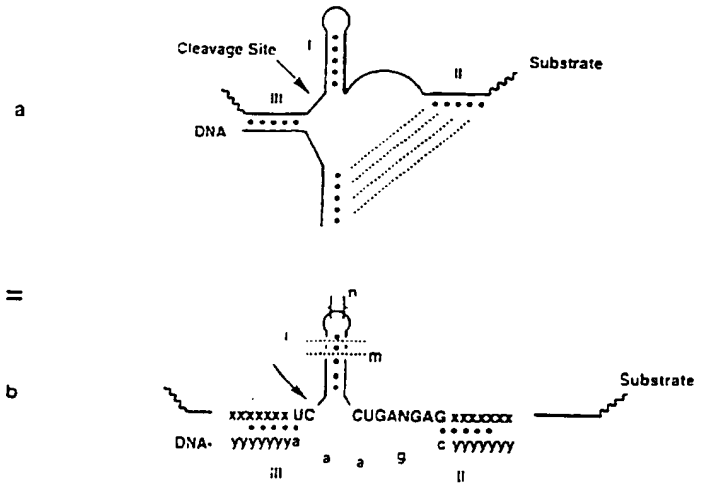
(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CATALYTIC DNA



(57) Abstract

Nucleic acid able to cause specific cleavage of a bond between two ribonucleotides in an RNA-containing molecule. The RNA-containing molecule has the structure: 5'-XnUHZCUGANGAGYm-3', wherein each X and Y is independently any nucleotide base; n and m are independently between 5 and 40; H is U, A or C; Z is a hairpin loop, having between 6 and 60 bases, and each U, C, G and A is a uracil, cytosine, guanosine, or adenosine-containing ribonucleotide, respectively, and N is any ribonucleotide. The nucleic acid has the structure: 3'-X'nM$_o$Y'm-5', wherein each X' and Y' are complementary nucleotide bases to each corresponding X and Y, and M$_o$ is a series of nucleotide bases active to cause the cleavage, and wherein M$_o$ contains no ribonucleotides.

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CATALYTIC DNA

Background of the Invention

This invention relates to the cleavage of RNA or DNA by a nucleic acid molecule.

The following is a brief history of the discovery and activity of enzymatic RNA molecules or ribozymes. This history is not meant to be complete but is provided only for understanding of the invention that follows. This summary is not an admission that all of the work described below is prior art to the claimed invention.

Prior to the 1970s it was thought that all genes were direct linear representations of the proteins that they encoded. This simplistic view implied that all genes were like ticker tape messages, with each triplet of DNA "letters" representing one protein "word" in the translation. Protein synthesis occurred by first transcribing a gene from DNA into RNA (letter for letter) and then translating the RNA into protein (three letters at a time). In the mid 1970s it was discovered that some genes were not exact, linear representations of the proteins that they encode. These genes were found to contain interruptions in the coding sequence which were removed from, or "spliced out" of, the RNA before it became translated into protein. These interruptions in the coding sequence were given the name of intervening sequences (or introns) and the process of removing them from the RNA was termed splicing. After the discovery of introns, two questions immediately arose: (i) why are introns present in genes in the first place, and (ii) how do they get removed from the RNA prior to protein synthesis? The first question is still being debated, with no clear answer yet available. The second question, how introns get removed from the RNA, is much better understood after a decade and a half of intensive research on this question. At least three different mechanisms have been discovered for removing introns from RNA. Two of these splicing mechanisms involve the binding of multiple protein factors which then act to correctly cut

and join the RNA. A third mechanism involves cutting and joining of the RNA by the intron itself, in what was the first discovery of catalytic RNA molecules.

Cech and colleagues were trying to understand how RNA splicing was accomplished in a single-celled pond organism called *Tetrahymena thermophila*. They had chosen *Tetrahymena thermophila* as a matter of convenience, since each individual cell contains over 10,000 copies of one intron-containing gene (the gene for ribosomal RNA). They reasoned that such a large number of intron-containing RNA molecules would require a large amount of (protein) splicing factors to remove the introns quickly. Their goal was to purify these hypothesized splicing factors and to demonstrate that the purified factors could splice the intron-containing RNA *in vitro*. Cech rapidly succeeded in getting RNA splicing to work *in vitro*, but something unexpected was occurring. As expected, splicing occurred when the intron-containing RNA was mixed with protein-containing extracts from *Tetrahymena*, but splicing also occurred when the protein extracts were left out. Cech proved that the intervening sequence RNA was acting as its own splicing factor to snip itself out of the surrounding RNA. They published this startling discovery in 1982. Continuing studies in the early 1980's served to elucidate the complicated structure of the *Tetrahymena* intron and to decipher the mechanism by which self-splicing occurs. Many research groups helped to demonstrate that the specific folding of the *Tetrahymena* intron is critical for bringing together the parts of the RNA that will be cut and spliced. Even after splicing is complete, the released intron maintains its catalytic structure. As a consequence, the released intron is capable of carrying out additional cleavage and splicing reactions on itself (to form intron circles). By 1986, Cech was able to show that a shortened form of the *Tetrahymena* intron could carry out a variety of cutting and joining reactions on other pieces of RNA. The demonstration proved that the *Tetrahymena* intron can act as a true enzyme: (i) each intron molecule was able to cut many substrate molecules while the intron molecule remained unchanged, and (ii) reactions were specific for RNA molecules that contained a unique sequence (CUCU) which allowed the intron to recognize and bind the RNA. Zaug and Cech coined the term "ribozyme" to describe any ribonucleic acid molecule that has enzyme-like properties. Also in 1986, Cech showed that the RNA substrate sequence

recognized by the *Tetrahymena* ribozyme could be changed by altering a sequence within the ribozyme itself. This property has led to the development of a number of site-specific ribozymes that have been individually designed to cleave at other RNA sequences. The *Tetrahymena* intron is the most well-studied of what is now recognized as a large class of introns, Group I introns. The overall folded structure, including several sequence elements, is conserved among the Group I introns, as is the general mechanism of splicing. Like the *Tetrahymena* intron, some members of this class are catalytic, *i.e.*, the intron itself is capable of the self-splicing reaction. Other Group I introns require additional (protein) factors, presumably to help the intron fold into and/or maintain its active structure. While the *Tetrahymena* intron is relatively large, (413 nucleotides) a shortened form of at least one other catalytic intron (SunY intron of phage T4, 180 nucleotides) may prove advantageous not only because of its smaller size but because it undergoes self-splicing at an even faster rate than the *Tetrahymena* intron.

Ribonuclease P (RNaseP) is an enzyme comprised of both RNA and protein components which are responsible for converting precursor tRNA molecules into their final form by trimming extra RNA off one of their ends. RNaseP activity has been found in all organisms tested, but the bacterial enzymes have been the most studied. The function of RNaseP has been studied since the mid-1970s by many labs. In the late 1970s, Sidney Altman and his colleagues showed that the RNA component of RNaseP is essential for its processing activity; however, they also showed that the protein component was also required for processing under their experimental conditions. After Cech's discovery of self-splicing by the *Tetrahymena* intron, the requirement for both protein and RNA components in RNaseP was reexamined. In 1983, Altman and Pace showed that the RNA was the enzymatic component of the RNaseP complex. This demonstrated that an RNA molecule was capable of acting as a true enzyme, processing numerous tRNA molecules without itself undergoing any change. The folded structure of RNaseP RNA has been determined, and while the sequence is not strictly conserved between RNAs from different organisms, this higher order structure is. It is thought that the protein component of the RNaseP complex may serve to stabilize the folded RNA *in vivo*. At least one RNA position important both to

substrate recognition and to determination of the cleavage site has been identified, however little else is known about the active site. Because tRNA sequence recognition is minimal, it is clear that some aspect(s) of the tRNA structure must also be involved in substrate recognition and cleavage activity. The size of RNaseP RNA (>350 nucleotides), and the complexity of the substrate recognition, may limit the potential for the use of an RNaseP-like RNA in therapeutics. However, the size of RNaseP is being trimmed down (a molecule of only 290 nucleotides functions reasonably well). In addition, substrate recognition has been simplified by the recent discovery that RNaseP RNA can cleave small RNAs lacking the natural tRNA secondary structure if an additional RNA (containing a "guide" sequence and a sequence element naturally present at the end of all tRNAs) is present as well.

Symons and colleagues identified two examples of a self-cleaving RNA that differed from other forms of catalytic RNA already reported. Symons was studying the propagation of the avocado sunblotch viroid (ASV), an RNA virus that infects avocado plants. Symons demonstrated that as little as 55 nucleotides of the ASV RNA was capable of folding in such a way as to cut itself into two pieces. It is thought that *in vivo* self-cleavage of these RNAs is responsible for cutting the RNA into single genome-length pieces during viral propagation. Symons discovered that variations on the minimal catalytic sequence from ASV could be found in a number of other plant pathogenic RNAs as well. Comparison of these sequences revealed a common structural design consisting of three stems and loops connected by central loop containing many conserved (invariant from one RNA to the next) nucleotides. The predicted secondary structure for this catalytic RNA reminded the researchers of the head of a hammer consisting of three double helical domains, stems I, II and III and a catalytic core (Figure 1a); thus it was named as such. Uhlenbeck was successful in separating the catalytic region of the ribozyme from that of the substrate. Thus, it became possible to assemble a hammerhead ribozyme from 2 (or 3) small synthetic RNAs. A 19-nucleotide catalytic region and a 24-nucleotide substrate, representing division of the hammerhead domain along the axes of stems I and II (Figure 1b) were sufficient to support specific cleavage. The catalytic domain of numerous hammerhead ribozymes have now been studied by both the Uhlenbeck and

Symons groups with regard to defining the nucleotides required for specific assembly and catalytic activity and determining the rates of cleavage under various conditions.

Haseloff and Gerlach showed it was possible to divide the domains of the hammerhead ribozyme in a different manner, division of the hammerhead domain along the axes of stems I and III (see, Figure 1c). By doing so, they placed most of the required sequences in the strand that didn't get cut (the ribozyme) and only required a UH where H=C, A, U in the strand that did get cut (the substrate). This resulted in a catalytic ribozyme that could cleave a UH-containing RNA sequence embedded within a longer "substrate recognition" sequence. The specific cleavage of a long mRNA, in a predictable manner using several such hammerhead ribozymes, was reported in 1988. A further development was the division of the catalytic hammerhead domain along the axes of stems III and II (Figure 1d, Jeffries and Symons, *Nucl. Acids. Res.* **1989**, *17*:1371.)

Deoxyribonucleotide substitutions in the fragment corresponding to construct 1c have been described by Yang et al., *Biochemistry* **1990**, *29*:11156; Perreault et al., *Biochemistry* **1991**, *30*:4020; Yang et al., *Biochemistry* **1992**, *31*:5005; Usman and Cedergren, *Trends in Biochem. Sci.* **1992**, *17*:334; Usman *et al.*, International Publication No. WO 92/07065; Eckstein et al., International Publication No. WO 93/15187; and Rossi *et al.*, *U.S. Patent* 5,149,796. Jeffries and Symons, *supra*, indicated that DNA used in construct **1d** does not catalyze the cleavage of RNA.

One plant pathogen RNA (from the negative strand of the tobacco ringspot virus) undergoes self-cleavage but cannot be folded into the consensus hammerhead structure described above. Bruening and colleagues have independently identified a 50-nucleotide catalytic domain for this RNA. In 1990, Hampel and Tritz succeeded in dividing the catalytic domain into two parts that could act as substrate and ribozyme in a multiple-turnover, cutting reaction. As with the hammerhead ribozyme, the hairpin catalytic portion contains most of the sequences required for catalytic activity while only a short sequence (GUC in this case) is required in the target. Hampel and Tritz described the folded structure of this RNA as consisting of a

single hairpin and coined the term "hairpin" ribozyme (Bruening and colleagues use the term "paper clip" for this ribozyme motif). Continuing experiments indicate an increasing number of similarities between the hairpin and hammerhead ribozymes in respect to both binding of target RNA and mechanism of cleavage. At the same time, the minimal size of the hairpin ribozyme is still 50-60% larger than the minimal hammerhead ribozyme.

Hepatitis Delta Virus (HDV) is a virus whose genome consists of single-stranded RNA. A small region (about 80 nucleotides) in both the genomic RNA, and in the complementary anti-genomic RNA, is sufficient to support self-cleavage. As the most recently discovered ribozyme, HDV's ability to self-cleave has only been studied for a few years, but is interesting because of its connection to a human disease. In 1991, Been and Perrotta proposed a secondary structure for the HDV RNAs that is conserved between the genomic and anti-genomic RNAs and is necessary for catalytic activity. Separation of the HDV RNA into "ribozyme" and "substrate" portions has recently been achieved by Been. Been has also succeeded in reducing the size of the HDV ribozyme to about 60 nucleotides.

The table below lists some of the characteristics of the ribozymes discussed above:

TABLE 1

Characteristics of Ribozymes

Group I Introns
Size: ~300 to >1000 nucleotides.
Requires a U in the target sequence immediately 5' of the cleavage site.
Binds 4-6 nucleotides at the 5'-side of the cleavage site.
Over 75 known members of this class. Found in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.

RNaseP RNA (M1 RNA)
Size: ~290 to 400 nucleotides.
RNA portion of a ribonucleoprotein enzyme. Cleaves tRNA precursors to form mature tRNA.
Roughly 10 known members of this group all are bacterial in origin.

Hammerhead Ribozyme
Size: ~30 to 40 nucleotides.
Requires the target sequence UH immediately 5' of the cleavage site.
Binds a variable number nucleotides on both sides of the cleavage site.
14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent (Figure 1)

Hairpin Ribozyme
Size: ~50 nucleotides.
Requires the target sequence GUC immediately 3' of the cleavage site.
Binds 4 nucleotides at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.
Only 1 known member of this class. Found in one plant pathogen (satellite RNA of the tobacco ringspot virus) which uses RNA as the infectious agent (Figure 5).

Hepatitis Delta Virus (HDV) Ribozyme
Size: ~60 nucleotides (at present).
Cleavage of target RNAs recently demonstrated.
Sequence requirements not fully determined.
Binding sites and structural requirements not fully determined, although no sequences 5' of the cleavage site are required.
Only 1 known member of this class. Found in human HDV (Figure 6).

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

Summary of the Invention

This invention concerns the cleavage of RNA by catalytic DNA or non-RNA-containing DNA-chimeras referred to as *Deoxyribozymes*, specifically cleavage by small deoxyribozymes, such as those which (together with their target RNA) form the motif referred to as hammerhead, hairpin, or hepatitis D virus. This activity in such deoxyribozymes is achieved by chemical synthesis of a deoxyribozyme.

Specifically, we describe the use of a construct similar to Figure 1d as a general scheme for nucleic acid able to cleave RNA. More specific examples are shown in Figures 2a and 2b, and a general scheme of the substrate repertoire available for such nucleic acid is shown in Figure 3.

A specific example of the DNA catalyzed cleavage of a 33-mer RNA strand (S) to a 7-mer product (P) is shown in Figure 4. The ratio of catalytic activity of the DNA catalyzed cleavage (lane 6) *vs* the RNA catalyzed (lane 4) and DNA-RNA chimera (lane 2) is about 1/10/8. Lanes 1, 3 and 5 show the same reactions following incubation of the enzymes with 10 mM NaOH at

80°C. As expected, the enzymatic activities of the ribozyme (lane 3) and the chimera (lane 1) are abolished, whereas the catalytic activity of the DNA only nucleic acid (lane 5) is maintained. We further show that other non-RNA-containing fragments also catalyze the RNA cleavage reaction.

As the term is used in this application, catalytic DNA enzymes, or *Deoxyribozymes*, are DNA, or non-RNA-containing DNA chimera molecules having an enzymatic activity which is able to cleave (preferably, repeatedly cleave) separate RNA molecules in a nucleotide base sequence specific manner.

Deoxyribozymes act by first binding to a target RNA. Such binding occurs through the DNA binding portion of a deoxyribozyme which is held in close proximity to the RNA (provided by the substrate) which acts to cleave the target RNA. Thus, the deoxyribozyme first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After a deoxyribozyme has bound and cleaved its RNA target it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

By the phrase "enzymatic DNA molecule" is meant a DNA molecule which has complementarity in a substrate binding region to a specified gene target, and also is able to cause specific cleavage of RNA in that target. That is, the enzymatic DNA molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. This complementarity functions to allow sufficient hybridization of the DNA molecule to the target RNA to allow the cleavage to occur. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention.

In preferred embodiments of this invention, the enzymatic DNA molecule, in conjunction with its target RNA, is formed in a hammerhead motif, but may also be formed in the motif of a hairpin or hepatitis delta virus. Examples of such hammerhead motifs are described by Rossi *et al.*, *Aids Research and Human Retroviruses* **1992**, *8*:183; of hairpin motifs by Hampel

et al., "RNA Catalyst for Cleaving Specific RNA Sequences," filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100 filed September 20, 1988, Hampel and Tritz, *Biochemistry* **1989**, *28*:4929, and Hampel *et al.*, *Nucleic Acids Research* **1990**, *18*:299; and an example of the hepatitis delta virus motif is described by Perrotta and Been, *Biochemistry* **1992**, *31*:16. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in a DNA molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within that substrate binding site which impart an RNA cleaving activity to the molecule.

The invention provides a method for producing a class of enzymatic cleaving agents that exhibit a high degree of specificity for the RNA of a desired target. The deoxyribozyme molecule is preferably targeted to a highly conserved sequence region of a target such that specific treatment of a disease or condition can be provided with a single deoxyribozyme. Such enzymatic DNA molecules can be delivered exogenously to specific cells as required. The preferred DNA molecule is small in size (less than 30 nucleotides, preferably between 13-20 nucleotides in length) so that the molecule allows the cost of treatment to be reduced compared to other ribozyme motifs.

Synthesis of ribozymes greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small deoxyribozyme motifs (*e.g.*, of the hammerhead structure) are used for exogenous delivery. The simple structure of these molecules increases the ability of the deoxyribozyme to invade targeted regions of the mRNA structure. Unlike the situation when the hammerhead structure is included within longer transcripts, there are no non-deoxyribozyme flanking sequences to interfere with correct folding of the deoxyribozyme structure or with complementary regions.

Thus, in a first aspect, the invention features nucleic acid able to cause specific cleavage of a bond between two ribonucleotides in an RNA molecule. The RNA molecule has the structure:

5'-XnUHZCUGANGAGYm-3' (SEQ. ID. NO. 1)

wherein, each X and Y is independently any nucleotide base; n and m are independently between 5 and 40; H is U, A or C; Z is a hairpin loop, having between 1 and 60 bases, preferably between 6 and 30 bases, and each U, C, G and A is a uracil, cytosine, guanosine, or adenosine-containing ribonucleotide, respectively, and N is any ribonucleotide. The nucleic acid has the structure:

3'-X'nM$_O$Y'm-5'

wherein, each X' and Y' are complementary nucleotide bases to each corresponding X and Y, and M$_O$ is a series of nucleotide bases (o in number) active to cause the cleavage. M$_O$ contains no ribonucleotides.

In preferred embodiments, M$_O$ is 3'-aaagc-5', wherein a, g and c are adenosine, guanosine and cytosine-containing deoxyribonucleotides, respectively; M$_O$ has at least one non-deoxynucleotide base; M$_O$ has all deoxyribonucleotide bases; X', M$_O$ and Y' have all deoxyribonucleotide bases; and M$_O$ has between 5 and 10 bases. Most preferably, the RNA-containing molecule has the structure:

5'-XnUCACCAGGAAACUGGUCUGANGAGYm-3' (SEQ. ID. NO. 2)

or 5'-XnUCUCCAGGAAACUGGACUGANGAGYm-3' (SEQ. ID. NO. 3)

and the nucleic acid has the structure:

3'-gcctcaaagcagtg-5' (SEQ. ID. NO. 4)

wherein, g, c, t and a are guanosine, cytosine, thymine and adenosine-containing deoxyribonucleotides.

In a second aspect, the invention features a method for causing specific cleavage of a bond between two nucleotides in an RNA-containing molecule having the structure shown above in Seq. ID. No. 1, including the steps of contacting the RNA-containing molecules with nucleic acid having the structure:

3'-X'nM$_O$Y'm-5'

wherein, each X' and Y' are complementary nucleotide bases to each corresponding X and Y, and M$_O$ is a series of nucleotide bases (o in number) active to cause the cleavage, under bond-cleaving conditions.

By "hairpin loop" is simply meant a structure which does not interfere with cleavage by the deoxyribozyme which generally contains about 4 to 7 base paired nucleotides with a small loop of between 4 and 8 nucleotides. Specifically, referring to Figure 3, m in that figure is between 4 and 8, and n in that figure is between 4 and 8.

Applicant is the first to discover that completely non-ribonucleotide-containing molecules can be used to cause specific cleavage of RNA-containing molecules in a catalytic or enzymatic fashion. This contrasts with the work of Jeffries and Symons who were unable to achieve this result. While an example in the hammerhead motif is provided below, those in the art will recognize that equivalent hairpin and hepatitis delta virus constructs can be readily formed. Optimization of a desirable deoxyribonucleotide deoxyribozyme can be achieved using standard methodology, and *in vitro* selection protocols can be readily devised to select optimum DNA molecules having such cleavage causing activity.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings:

Figure 1a is a diagrammatic representation of the hammerhead ribozyme domain known in the art; Figure 1b is a diagrammatic representation of the hammerhead ribozyme as divided by Uhlenbeck into a substrate and enzyme portion; Figure 1c is a similar diagram showing the hammerhead

divided by Haseloff and Gerlach into two portions: and Figure 1d is a similar diagram showing the hammerhead divided by Jeffries and Symons into two portions.

Figures 2a and 2b are representations of specific examples of the cleavage of RNA by a catalytic DNA strand in the hammerhead domain.

Figure 3 is a representation of the general structure of any substrate RNA molecule able to participate in the DNA mediated catalysis of RNA cleavage.

Figure 4 is an autoradiogram showing the cleavage of an RNA substrate by a catalytic DNA strand of the structure shown in Figure 2.

Specifically: Autoradiogram of a 20% polyacrylamide/7 M urea gel of ^{32}P 5'-end labeled RNA substrate (**S**) cleavage reactions (P = product). Cleavage by deoxyribozyme (**D**), chimeric nucleozyme (**N**) and standard ribozyme (**R**) molecules were at 30°C in 10 mM Mg^{2+} with [E]/[S] = 1 unless otherwise noted. A + indicates the addition of NaOH. Lanes: 1) **S** + **N** + NaOH, 2) **S** + **N** , 3) **S** + **R** + NaOH, 4) **S** + **R**, 5) **S** + **D** + NaOH, 6) **S** + **D**. The positions of xylene cyanol FF dye (XC) and bromophenol blue dye (BP) are indicated.

Figure 5 is a representation of general structure of the hairpin ribozyme domain known in the art.

Figure 6 is a representation of general structure of the hepatitis delta virus ribozyme domain known in the art.

Figure 7 is a representation of a selection protocol for other catalytic substrate domains for deoxyribozymes.

RNA Cleaving Deoxyribozymes:

RNA cleaving deoxyribozymes useful in this invention are generally described above. These deoxyribozymes are generally formed from deoxyribonucleotides, but such deoxyribonucleotides can be modified from those occurring in nature to have different chemical substituents in the 2'

position (see, Usman, *supra*, and Eckstein, *supra*), as well as modified bases well known in the art.

Examples:

The following are non-limiting examples showing the synthesis of non-nucleotide mimetic containing catalytic nucleic acids using non-nucleotide phosphoramidites.

Example 1: Cleavage of 5'- CGG AGU CAC CAG GAA ACU GGU CUG AUG AGU CAC -3' by 5'- d(gtg acg aaa ctc cg) -3'

The substrate molecule (**S**) shown in Figure 2a was 5'-end labeled with ^{32}P and purified by PAGE. The cleavage of **S** was affected by a deoxyribozyme (**D**), 5'- d(gtg acg aaa ctc cg) -3' a nucleozyme (**N**), 5'- d(gtg acg aar(A) ctc cg) -3', and a ribozyme (**R**), 5'- GTG ACG AAA CTC CG -3' at 30°C in 10 mM Mg^{2+} with [E]/[S] = 1 (lanes 6, 2 and 4, respectively, in Figure 4). As a control for the catalytic cleavage by the deoxyribozyme, all three enzymes were pre-treated with 10 mM NaOH at 80°C (lanes 5, 1 and 3, respectively, in Figure 4). This pre-treatment cleaves any RNA-containing enzyme and thereby abolishes its enzymatic activity. This was seen in the case of the nucleozyme (**N**) reaction, cleavage in lane 2 was abolished in lane 1, and in the case of the ribozyme (**R**) reaction, cleavage in lane 4 was abolished in lane 3. In the case of the deoxyribozyme (**D**) reaction, cleavage in lane 6 was not abolished in lane 5. The ratio of catalytic activity of the DNA catalyzed cleavage (lane 6) *vs* the RNA catalyzed (lane 4) and DNA-RNA chimera (nucleozyme, lane 2) was about 1/10/8.

Example 2: Site Searches

The general utility of using the method of RNA cleavage is illustrated by a search of the HSV genome. The ~156,000 bp HSV genome was searched using GeneWorks_ V2.0 Software (IntelliGenetics, Mountain View, CA). The search parameter used was to find CUGANGAG. A total of eight sites were found, two of which were in the coding region. The site # from the 5'-end, gene product, coding or non-coding status and whether the site is in the + or - strand are listed below.

10068	UL2	Non-Coding	-
56919	UL28	Non-coding	+
65123	UL30	Non-Coding	-
90571	UL40	Non-Coding	-
93596	UL42	Coding	+
93650	UL42	Coding	+
101268	UL47	Non-Coding	+
143338	US9	Non-Coding	-

These sites are potentially useful targets for deoxyribozymes of the present invention. Other such searches can be performed using narrower search terms for the generic structure shown in Figure 3.

Example 3: *In vitro* Selection of New Motifs

To expand the repertoire of substrate motifs beyond the CUGANGAG example an *in vitro* selection strategy may be used to find other sites/motifs. This strategy is illustrated in Figure 7. In this strategy a synthetic substrate population **a** containing randomized bases at the positions denoted as "N", is subjected to cleavage with a catalytic DNA molecule (this population may be generated either by chemical synthesis of RNA containing randomized ribonucleotides "N" or by the chemical synthesis of DNA containing randomized deoxyribonucleotides "N" followed by transcription with T7 RNA polymerase). The resulting cleavage product population **b** is phosphorylated (p) to yield population **c**. This then allows for the ligation of a PCR primer site to the cleavage product population to yield a tagged population **d**. Conversion of the tagged RNA population **d** to DNA *via* reverse transcriptase followed by PCR of the resulting tagged DNA population gives a double-stranded DNA population **e** containing the sequences capable of supporting DNA catalyzed RNA cleavage. Population **e** is then ligated into a plasmid to give a closed circular double-stranded plasmid population **f**. Subjecting plasmid population **f** to cleavage by the restriction endonuclease *Eco*57 i, cleavage sequence 5'- d(CTGAAGA) -3', yields two populations of molecules **g** and **h**. Population **g** contains all wild-type sequences while population **h** contains new motif sequences. The new motif sequences can then be

identified by cloning and sequencing population **h**. This method can be varied by changing the number of "N" nucleotides in population **a**.

Other embodiments are within the following claims.

"Sequence Listing"

(1) GENERAL INFORMATION:

(i) APPLICANT: USMAN, Nassim
CEDERGREN, Robert J.
CHARTRAND, Pascal
HARVEY, Stephen

(ii) TITLE OF INVENTION: CATALYTIC DNA

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Lyon & Lyon
(B) STREET: 611 West Sixth Street
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 90017

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

Prior applications total, including application described below: none

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: WARBURG, Richard J.
(B) REGISTRATION NUMBER: 32,327
(C) REFERENCE/DOCKET NUMBER: 203/152

(ix) TELECOMMUNICATION INFORMATION:

(A)	TELEPHONE:	(213) 489-1600
(B)	TELEFAX:	(213) 955-0440
(C)	TELEX:	67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	11
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION: The letter "Z" represents a hairpin loop; the letter "N" stands for U, C, G or A; the letter "H" stands for U, A or C

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

5' UHZCUGANGA G 3' 11

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	24
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION: The letter "N" stands for U, C, G or A.

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5' UCACCAGGAA ACUGGUCUGA NGAG 3' 24

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	24
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION: The letter "N" stands for U, C, G or A.

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

5' UCUCCAGGAA ACUGGACUGA NGAG 3' 24

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	14
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

3' GCCTCAAAGC AGTG 5' 14

Claims

1. Nucleic acid able to cause specific cleavage of a bond between two ribonucleotides in an RNA-containing molecule having the structure:

5'-XnUHZCUGANGAGYm-3'

wherein each X and Y is independently any nucleotide base; n and m are independently between 5 and 40; H is C, A or U; Z is a hairpin loop, comprising between 6 and 60 bases, and each U, C, G and A is a uracil, cytosine, guanosine, or adenosine-containing ribonucleotide, respectively, and N is any ribonucleotide; wherein said nucleic acid has the structure:

3'-X'nM$_O$Y'm-5'

wherein each X' and Y' are complementary nucleotide bases to each corresponding X and Y, and M$_O$ is a series of nucleotide bases active to cause said cleavage; wherein M$_O$ contains no ribonucleotides.

2. The nucleic acid of claim 1, wherein M$_O$ is 3'-aaagc-5', wherein a, g and c are adenosine, guanosine and cytosine-containing deoxyribonucleotides, respectively.

3. The nucleic acid of claim 1, wherein said RNA-containing molecule has the structure chosen from:

5'-XnUCACCAGGAAACUGGUCUGANGAGYm-3' and

5'-XnUCUCCAGGAAACUGGACUGANGAGYm-3'

4. The nucleic acid of claim 3, wherein said nucleic acid has the structure:

3'-gcctcaaagcagtg-5'

wherein, g, c, t and a are guanosine, cytosine, thymine and adenosine-containing deoxyribonucleotides.

5. The nucleic acid of claim 1, wherein M_O comprises at least one non-deoxynucleotide base.

6. The nucleic acid of claim 1, wherein M_O comprises all deoxyribonucleotide bases.

7. The nucleic acid of claim 1, wherein X', M_O and Y' comprise all deoxyribonucleotide bases.

8. The nucleic acid of claim 1, wherein M_O comprises between 5 and 10 bases.

9. Method for causing specific cleavage of a bond between two nucleotides in an RNA-containing molecule having the structure:

5'-XnUHZCUGANGAGYm-3'

wherein each X and Y is independently any nucleotide base; n and m are independently between 5 and 40; H is C, U or A; Z is a hairpin loop, comprising between 6 and 60 bases, and each U, C, G and A is a uracil, cytosine, guanosine, or adenosine-containing ribonucleotide, respectively, and N is any ribonucleotide, comprising the steps of contacting the RNA-containing molecule with nucleic acid having the structure:

3'-X'nM_OY'm-5'

wherein each X' and Y' are complementary nucleotide bases to each corresponding X and Y, and M_O is a series of nucleotide bases active to cause the cleavage, under bond-cleaving conditions.

10. The method of claim 9, wherein M_O is 3'-aaagc-5', wherein a, g and c are adenosine, guanosine and cytosine-containing deoxyribonucleotides, respectively.

11. The method of claim 9, wherein said RNA-containing molecule has the structure chosen from:

5'-XnUCACCAGGAAACUGGUCUGANGAGYm-3' and

5'-XnUCUCCAGGAAACUGGACUGANGAGYm-3'

12. The method of claim 11, wherein said nucleic acid has the structure:

3'-gcctcaaagcagtg-5'

wherein, g, c, t and a are guanosine, cytosine, thymine and adenosine-containing deoxyribonucleotides.

13. The method of claim 9, wherein M_O comprises at least one non-deoxynucleotide base.

14. The method of claim 9, wherein M_O comprises all deoxyribonucleotide bases.

15. The method of claim 9, wherein X', M_O and Y' comprise all deoxyribonucleotide bases.

16. The method of claim 9, wherein M_O comprises between 5 and 10 bases.

Abstract of the Invention

Nucleic acid able to cause specific cleavage of a bond between two ribonucleotides in an RNA-containing molecule. The RNA-containing molecule has the structure:

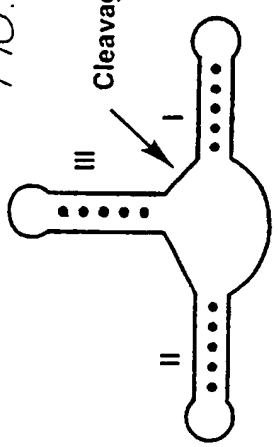
5'-XnUHZCUGANGAGYm-3'

wherein each X and Y is independently any nucleotide base; n and m are independently between 5 and 40; H is U, A or C; Z is a hairpin loop, having between 6 and 60 bases, and each U, C, G and A is a uracil, cytosine, guanosine, or adenosine-containing ribonucleotide, respectively, and N is any ribonucleotide. The nucleic acid has the structure:

3'-X'nM$_O$Y'm-5'

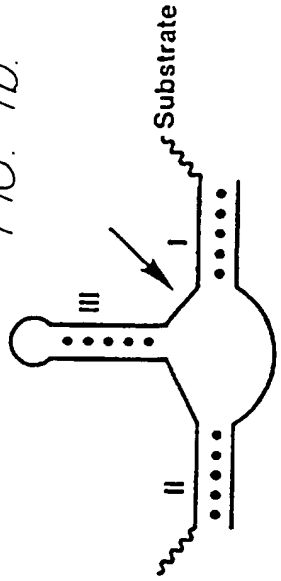
wherein each X' and Y' are complementary nucleotide bases to each corresponding X and Y, and M$_O$ is a series of nucleotide bases active to cause the cleavage, and wherein M$_O$ contains no ribonucleotides.

FIG. 1a.



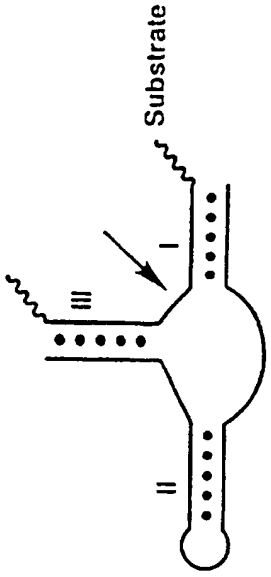
a

FIG. 1b.



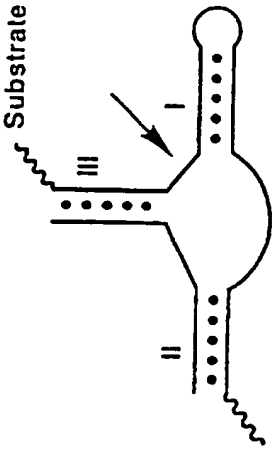
b

FIG. 1c.



c

FIG. 1d.



d

FIG. 2a

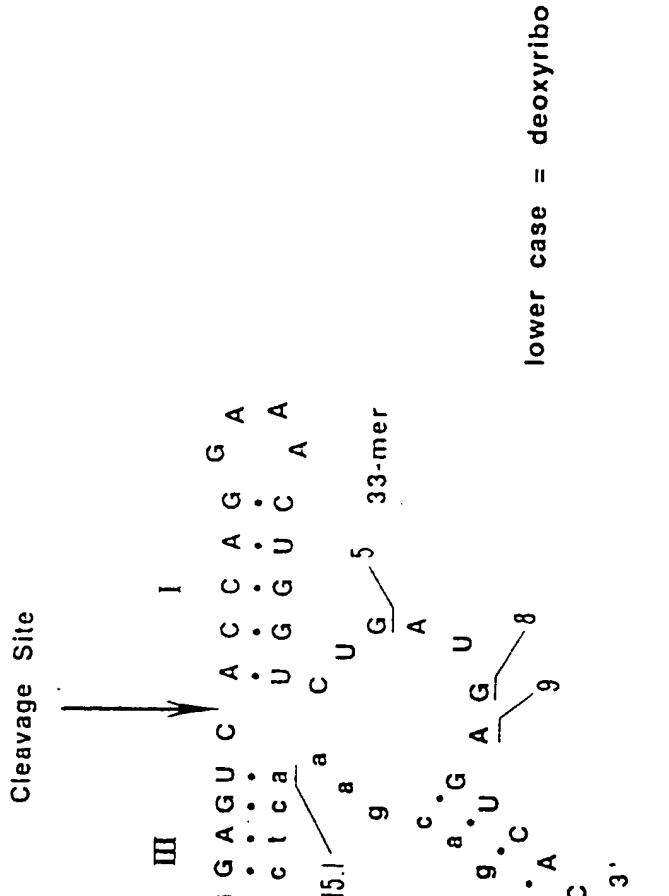
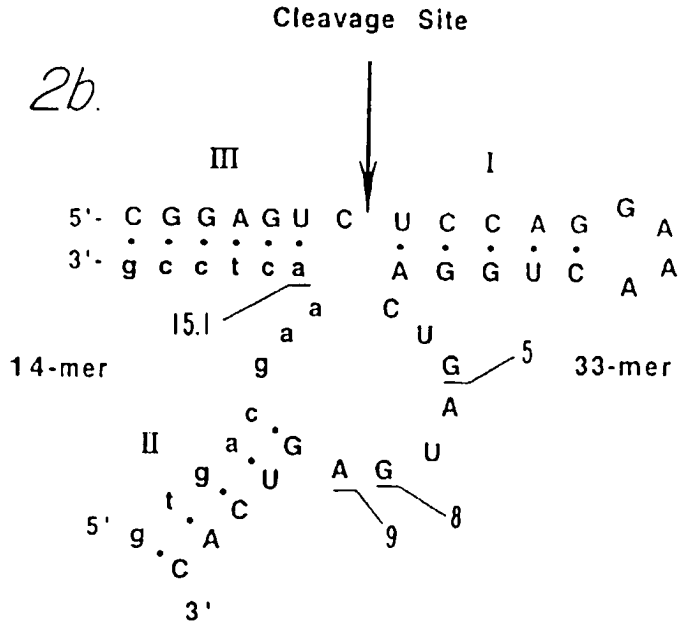

Cleavage Site
I
III
II
5'- C G G A G U C A C C A G G A
3'- g c c t c a U G G U C A A
14-mer
33-mer
lower case = deoxyribo
RNA Substrate: 5'- CGG AGU CAC CAG GAA ACU GGU CUG AUG AGU CAC -3'
DNA Catalyst: 5'- gtg acg aaa ctc cg -3' = Deoxyribozyme
DNA/RNA Catalyst: 5'- gtg acg aaA ctc cg -3' = Nucleozyme
RNA Catalyst: 5'- GTG ACG AAA CUC CG -3' = Ribozyme

FIG. 2b.



lower case = deoxyribo

RNA Substrate:	5'- CGG AGU CUC CAG GAA ACU GGA CUG AUG AGU CAC -3'	
DNA Catalyst:	5'- gtg acg aaa ctc cg -3'	= Deoxyribozyme
DNA/RNA Catalyst:	5'- gtg acg aaA ctc cg -3'	= Nucleozyme
RNA Catalyst:	5'- GTG ACG AAA CUC CG -3'	= Ribozyme

FIG. 3a.

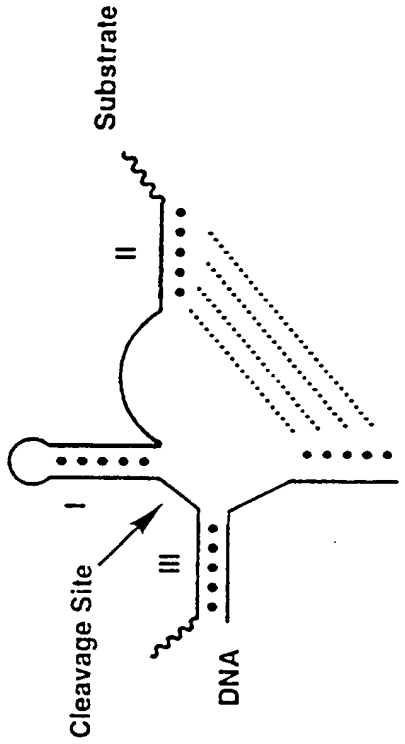
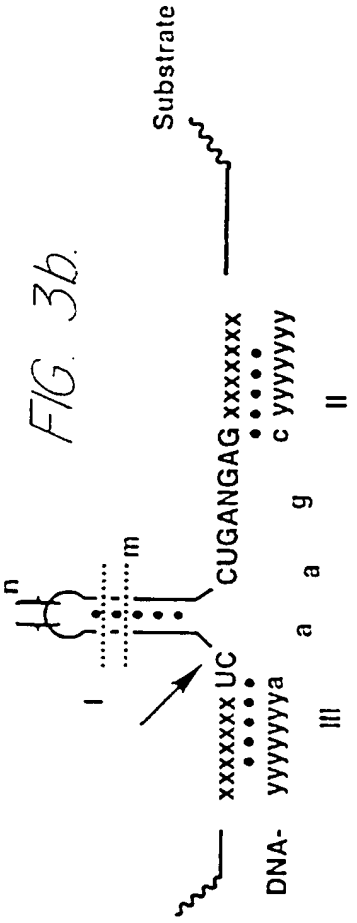


FIG. 3b.



a II b

Figure 4
RNA Cleavage by a Deoxyribozyme

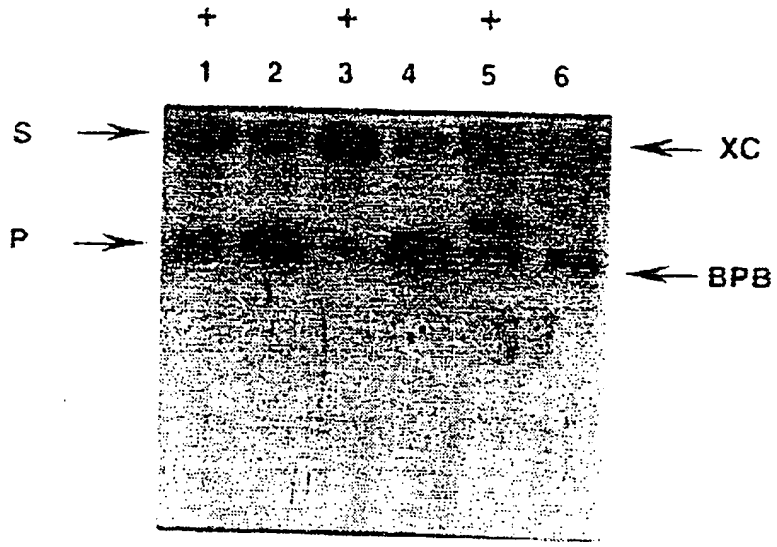
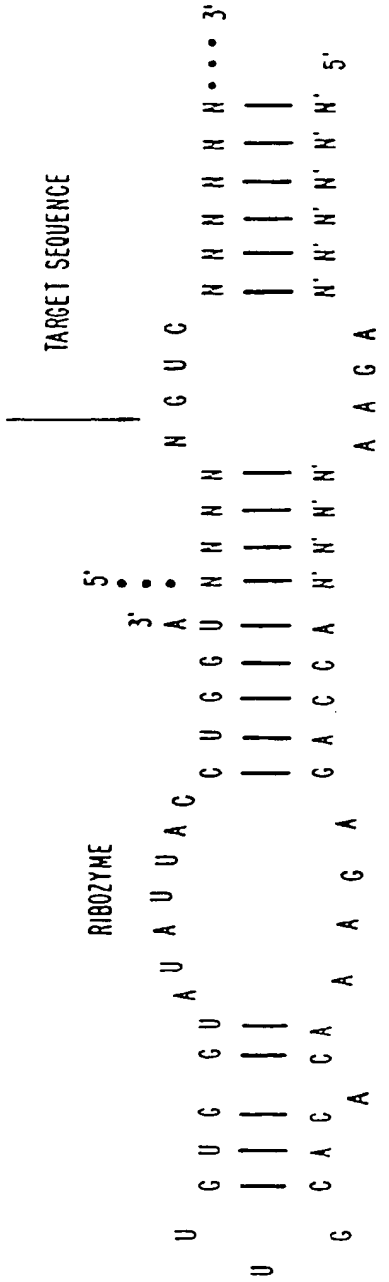


FIG. 5.


TARGET SEQUENCE
RIBOZYME
5'
3'
N G U C
N N N N N N N • • • 3'
N' N' N' N' N' N' 5'
A A G A
A U A U A C
C U G G U N N N N N
G A C C A N' N' N' N'
U G U C G U
A
U G C A C C A A A G A
A

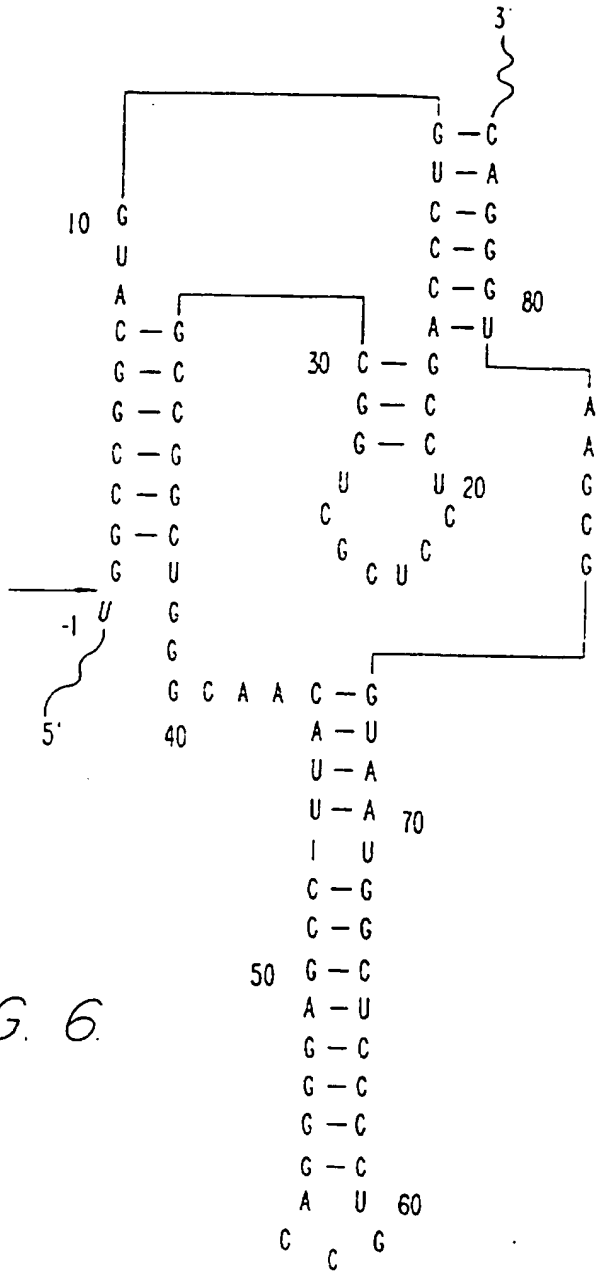
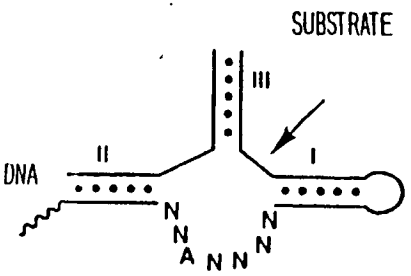
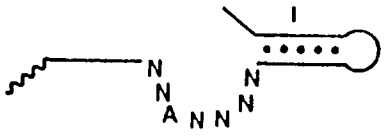


FIG. 6



CLEAVE

FIG. 7a.



PHOSPHORYLATE

FIG. 7b.

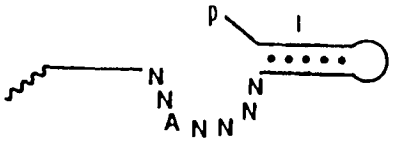


FIG. 7c.

LIGATE

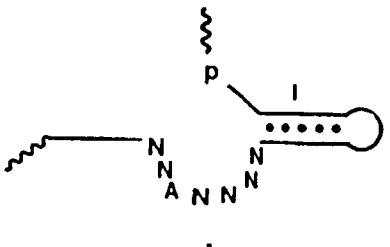


FIG. 7d.

1. RT

2. PCR

FIG. 7e.

LIGATE-PLASMID

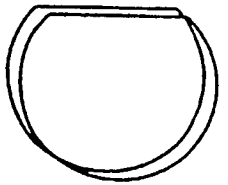
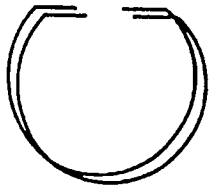


FIG. 7f

FIG. 7g.

FIG. 7h.

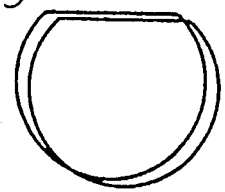
RESTFICTION ENDONUCLEASE CLEAVAGE OF CUGAAGA



d(CTGAAGA) CONTAINING

g

+



NEW MOTIF CONTAINING

h

CLONE & SEQUENCE

= PRIMER SITE

INTERNATIONAL SEARCH REPORT

Interna ti Application No
PCT/US 94/11649

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N9/00 C07H21/04 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 15187 (MASSACHUSETTS INST TECHNOLOGY) 5 August 1993 cited in the application see page 3, line 14 - line 17 see page 23, line 6 - line 7 see claims	1-16
A	WO,A,91 19789 (COMMW SCIENT IND RES ORG) 26 December 1991 see claims; example 2	1-16
A	WO,A,92 07065 (MAX PLANCK GESELLSCHAFT) 30 April 1992 cited in the application see page 4, paragraph 5 - page 6 see claims	1-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 1995	17.03.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 344, 5 April 1990 LONDON GB, pages 565-567, PERREAULT, J.-P. ET AL. 'Mixed deoxyribo- and ribooligonucleotides with catalytic activity' see the whole document	1-16
A	SCIENCE, vol. 236, 19 June 1987 LANCASTER, PA US, pages 1532-1539, CECH, T. 'The chemistry of self-splicing RNA and RNA enzymes' see page 1537, right column, line 16 - line 20	1
A	NUCLEIC ACIDS RESEARCH, vol. 21, no.11, 11 June 1993 ARLINGTON, VIRGINIA US, pages 2605-11, SHIMAYAMA, T. ET AL. 'Nuclease-resistant chimeric ribozymes containing deoxyribonucleotides and phosphorothioate linkages' see the whole document	1

1

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US94/11649

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Altough claims 9-16 (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/US 94/11649

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9315187	05-08-93	AU-B-	3600193	01-09-93
		CA-A-	2129119	05-08-93
		EP-A-	0625192	23-11-94
WO-A-9119789	26-12-91	AU-B-	650032	09-06-94
		AU-A-	7997691	07-01-92
		EP-A-	0535067	07-04-93
		JP-T-	5509224	22-12-93
		US-A-	5298612	29-03-94
WO-A-9207065	30-04-92	AU-B-	649074	12-05-94
		CA-A-	2093664	13-04-92
		DE-T-	552178	03-02-94
		EP-A-	0552178	28-07-93
		ES-T-	2061416	16-12-94
		JP-T-	6501842	03-03-94